Quorum-sensing signaling is required for production of the antibiotic pyrrolnitrin in a rhizospheric biocontrol strain ofSerratia plymuthica

Liu, Xiaoguang ; Bimerew, Mohammed ; Ma, Yingxin ; Müller, Henry ; Ovadis, Marianna ; Eberl, Leo ; Berg, Gabriele ; Chernin, Leonid

Abstract: One mechanism that bacteria have adopted to regulate the production of antimicrobial compounds is population-density-dependent LuxRI-type quorum sensing (QS), exploiting the production of N-acyl homoserine lactone (AHL) autoinducer signals. In biocontrol bacteria, most known cases involve the AHL control of phenazine antibiotics production by rhizospheric pseudomonads. This work is the first to demonstrate that phenazines are not the only group of biocontrol-related antibiotics whose production is regulated by QS systems. Strain HRO-C48 of Serratia plymuthica isolated from the rhizosphere of oilseed rape and described as a chitinolytic bacterium, which protects crops against Verticillium wilt, was also shown to produce wide-range antibiotic pyrrolnitrin and several AHLs, including N-butanoyl-HSL, N-hexanoyl-HSL and N-3-oxo-hexanoyl-HSL (OHHL). The genes splI and splR, which are analogues of luxI and luxR genes from other Gram-negative bacteria, were cloned and sequenced. The mutant AHL-4 (splI::miniTn5) was simultaneously deficient in the production of AHLs and pyrrolnitrin, as well as in its ability to suppress the growth of several fungal plant pathogens in vitro. However, pyrrolnitrin production could be restored in this mutant by introduction of the splIR genes cloned into a plasmid or by addition of the conditioned medium from strain C48 or OHHL standard to the growth medium

DOI: https://doi.org/10.1111/j.1574-6968.2007.00681.x

Quorum-sensing signaling is required for production of the antibiotic pyrrolnitrin in a rhizospheric biocontrol strain of *Serratia plymuthica*

Xiaoguang Liu[1,2], Mohammed Bimerew[3], Yingxin Ma[2], Henry Müller[4,5], Marianna Ovadis[3], Leo Eberl[6], Gabriele Berg[4,5] & Leonid Chernin[3]

[1]Institute of Life Sciences, Jiangsu University, Zhenjiang, Jiangsu, China; [2]Institute of Plant Pathology, Shandong Agricultural University, Taian, Shandong, China; [3]The Otto Warburg Minerva Centre for Agriculture Biotechnology, Hebrew University of Jerusalem, Rehovot, Israel; [4]Institute of Environmental Biotechnology, Graz University of Technology, Graz AT, Austria; [5]Institute of Microbiology, University of Rostock, Rostock, Germany; and [6]Institute of Microbiology, University of Zurich, Zurich, Switzerland

Correspondence: Leonid Chernin, The Otto Warburg Minerva Centre for Agriculture Biotechnology, Hebrew University of Jerusalem, Rehovot 46100, Israel. Tel.: +972 8 9489128; fax: +972 8 9468785; e-mail: chernin@agri.huji.ac.il

Received 21 November 2006; revised 25 January 2007; accepted 6 February 2007. First published online 13 March 2007.

DOI:10.1111/j.1574-6968.2007.00681.x

Editor: Hermann Bothe

Keywords
N-acyl homoserine lactones; plant growth-promoting rhizobacteria; antifungal compounds; insertion mutagenesis; biocontrol.

Abstract

One mechanism that bacteria have adopted to regulate the production of antimicrobial compounds is population-density-dependent LuxRI-type quorum sensing (QS), exploiting the production of *N*-acyl homoserine lactone (AHL) autoinducer signals. In biocontrol bacteria, most known cases involve the AHL control of phenazine antibiotics production by rhizospheric pseudomonads. This work is the first to demonstrate that phenazines are not the only group of biocontrol-related antibiotics whose production is regulated by QS systems. Strain HRO-C48 of *Serratia plymuthica* isolated from the rhizosphere of oilseed rape and described as a chitinolytic bacterium, which protects crops against *Verticillium* wilt, was also shown to produce wide-range antibiotic pyrrolnitrin and several AHLs, including *N*-butanoyl-HSL, *N*-hexanoyl-HSL and *N*-3-oxo-hexanoyl-HSL (OHHL). The genes *splI* and *splR*, which are analogues of *luxI* and *luxR* genes from other Gram-negative bacteria, were cloned and sequenced. The mutant AHL-4 (*splI*::miniTn5) was simultaneously deficient in the production of AHLs and pyrrolnitrin, as well as in its ability to suppress the growth of several fungal plant pathogens *in vitro*. However, pyrrolnitrin production could be restored in this mutant by introduction of the *splIR* genes cloned into a plasmid or by addition of the conditioned medium from strain C48 or OHHL standard to the growth medium.

Introduction

Expression of several phenotypic characteristics in bacteria, e.g. bioluminescence, biofilm formation, motility, production of virulence factors, exoenzymes and antibiotics, is often a cell-density-dependent phenomenon mediated by cell-to-cell communication in a process known as quorum sensing (QS). In Gram-negative bacteria, the main QS system, known as LuxIR, operates to control the response, mainly via production of *N*-acylated homoserine lactone (AHL) signaling (Waters & Bassler, 2005). Various plant-growth-promoting rhizobacteria (PGPR), which are able to protect plants from fungal and bacterial diseases, have been found to produce AHLs and other QS signals (Loh *et al.*, 2002; Haas & Defago, 2005). The production of antifungal metabolites and lytic enzymes, induction of systemic resistance, and high competitive maintenance in the rhizosphere are considered to be important prerequisites for the optimal performance of PGPR toward plant pathogens (Haas & Defago, 2005).

Antibiotics are major determinants of antagonism against fungi by various PGPR. Among the fluorescent pseudomonads, many strains produce one or more potent metabolites with antifungal activity. The best-characterized are simple compounds such as phenazines, 2,4-diacetylphloroglucinol, 3-chloro-4-(2′-nitro-3′-chlorophenyl)-pyrrole (pyrrolnitrin, Prn) and pyoluteorin (Raaijmakers *et al.*, 2002). However, only a few examples of the role of QS systems in the regulation of these secondary metabolites' production have been described. Most known cases involve the AHL control of phenazines antibiotics (Loh *et al.*, 2002; Chin-A-Woeng *et al.*, 2003).

Several plant-associated isolates of *Serratia* species, mainly *Serratia marcescens* and *Serratia plymuthica*, have been described as biocontrol agents for fungal plant pathogens (Berg, 2000, Kamensky *et al.*, 2003; Ovadis *et al.*, 2004). These isolates are known to produce a range of antifungal metabolites, including the antibiotics pyrrolnitrin and chlorinated macrolide haterumalides (Kalbe *et al.*, 1996; Frankowski *et al.*, 1998; Kamensky *et al.*, 2003; Levenfors *et al.*, 2004). At least four different LuxRI/AHL QS systems have been described in *Serratia* (Wei & Lai, 2006). These systems control population surface migration, biofilm development and production of the biosurfactant serrawettin, the antibiotics carbapenem and prodiogosin, chitinases, proteases and other exoenzymes (Eberl *et al.*, 1999; Thomson *et al.*, 2000; Horng *et al.*, 2002; Christensen *et al.*, 2003; Rice *et al.*, 2005). Additionally, LuxS/autoinducer-2 (AI-2) has been reported in *S. marcescens* as a second QS system shown to control some phenotypes, including production of the antibiotic prodigiosin, jointly with the LuxIR systems (Coulthurst *et al.*, 2004), while LuxS system described in the *S. plymuthica* strain RVH1 from a food-processing environment was shown participate in the bacteria growth regulation (Van Houdt *et al.*, 2006). However, much less is known about QS systems operating in rhizospheric *Serratia* isolates with the potential to serve as biocontrol agents of plant pathogens.

The main aim of this work was to investigate the role of AHL signaling in the expression of antifungal traits in rhizospheric bacteria, focusing on pyrrolnitrin production, which has only begun to be investigated with respect to QS regulation. *Serratia plymuthica* strain HRO-C48, characterized by a wide range of activities against plant-pathogenic fungi (Kalbe *et al.*, 1996; Frankowski *et al.*, 1998, 2001; Kurze *et al.*, 2001), was used as the model organism.

Materials and methods

Organisms, plasmids and growth media

Serratia plymuthica strain HRO-C48 (= DSMZ12502) was isolated from the rhizosphere of oilseed rape (*Brassica napus* L.) (Kalbe *et al.*, 1996). *Escherichia coli* strain S17-1 (Simon *et al.*, 1983) and its derivative carrying the pUT-miniTn5-Km2*lacZ* plasmid (de Lorenzo & Timmis, 1994) were kindly supplied by M. Winson (University of Wales, Aberystwyth, UK). AHL reporter strains *Chromobacterium violaceum* CV026, *E. coli*/pSB536 and *E. coli*/pSB401 were gifts from P. Williams (Nottingham University, Nottingham, UK), while the AHL reporter *Agrobacterium tumefaciens* NTL4/pZLR4 was kindly supplied by S. Farrand (University of Illinois at Urbana-Champaign, Urbana, Illinois). *Escherichia coli* strain DH5a carrying the broad-host-range plasmid vector pUCP26 was provided by D. Mavrodi (Washington State University, Pullman). The fungi were from the authors' laboratory collection. Liquid or solid (1.5% w/v agar) Luria–Bertani (LB) broth or LB agar (LA), M9 minimal-glucose medium (Ausubel *et al.*, 1994) and potato dextrose agar (PDA) (Difco, Sparks, MD) were used as growth media. *Escherichia coli* and *S. plymuthica* strains were grown at 37 and 30 °C, respectively. To detect pyrrolnitrin production, strains were grown in 925 minimal medium supplemented with 2% (w/v) glycerol (Ovadis *et al.*, 2004). Antibiotic supplements were used at the following concentrations: ampicillin (Amp), 100 μg mL^{-1}; rifampicin (Rif), 40 μg mL^{-1}; tetracycline (Tc), 20 μg mL^{-1}. All these antibiotics, as well as pyrrolnitrin standard, were purchased from Sigma.

AHL production and detection

The following AHL reporters were used: pigment violacein-reporter strain *Chromobacterium violaceum* CV026 (McClean *et al.*, 1997), two *E. coli* strains carrying recombinant plasmids pSB401 or pSB536 designed for *lux*-based reporter assays (Winson *et al.*, 1998) and *A. tumefaciens* NTL4/pZLR4(Shaw *et al.*, 1997). The procedures and reporter strains are described in detail in Rice *et al.* (2004). Briefly, ethyl-acetate extracts obtained from 50 mL of cell-free culture supernatant from bacterial cultures of the tested strains grown overnight to stationary phase in LB broth or M9 minimal-glucose medium were evaporated and dissolved in 50 μL of acetonitrile. Violacein production was indicated by dark blue/purple pigmentation of the bacterial lawn surrounding the wells made in the solidified top LA layer. Light emission was monitored using a CL-BIS luminometer (DNR Imaging Systems Ltd, Israel). Synthetic AHL standards (kindly supplied by P. Williams) were spotted on plates to confirm reporter-strain activity. For detection of the AHLs by thin-layer chromatography (TLC) analysis, AHLs crude extracts dissolved in acetonitrile and synthetic AHL standards were spotted onto glass-backed RP18 F_{254S} reverse-phase TLC plates (Merck, Germany) and samples were separated using 60% (v/v) methanol in water as the solvent. Then TLC plates were overlaid with the reporter strain seeded in 0.7% (v/v) LA in the case of reporter CV026. After overnight incubation at 30 °C, AHLs were located visually as dark blue/purple spots. TLC analysis was repeated at least three times with samples obtained independently for each assay.

DNA manipulations

Standard procedures (Ausubel *et al.*, 1994) were generally used. For detection of *prn* genes in strain C48, the primers to the *prnC* gene of the *prnABCD* operon of *Pseudomonas fluorescens* and PCR program described by Mavrodi *et al.* (2001) were used. PCR amplification was carried out in a

25-μL reaction mixture containing 100 ng of genomic DNA of strain C48, 200 μM of each dNTP, 10 pmol of each primer and 1 U of Taq DNA polymerase. The genomic DNA of mutant C48/AHL-4 (see below) was proved for the presence of miniTn5-Km2*lacZ* by PCR using primers *lacZ*-F2662 5′-GCAGGTAGCAGAGCGGGTAA and Km-R698 5′-ACT-CACCGAGGCAGT-TCCAT. The inverse PCR technique was used for mapping of pUT-miniTn5-Km2*lacZ* transposon insertion in the AHL-4 mutant generally as described by Ovadis *et al.* (2004). Briefly, the primers Km F696 5′-ATGGAACTGCCTCGGTGAGT and *lacZ* R159 TTCAGGC-TGCGCAACTGTT were designed to the known sequence of miniTn5-Km2lacZ in opposite orientations. Genomic DNA of the AHL-4 mutant was digested by restriction enzymes (BamHI, PstI, SacII, SalI, XbaI, XhoI), which did not cut the pUT-miniTn5-Km2*lacZ* transposon. Each cut genomic DNA was subjected to self-ligation and used for iPCR. PCR was performed in a total volume of 25 μL, containing 200 μM of each dNTP, 10 pmol of each PCR primer, 1 U of error-proof LA *Taq*^{TM} DNA polymerase (TaKaRa Biotechnology Co. Ltd, Dalian, China) and 100 ng genomic DNA from the AHL-4 mutant used as a matrix. The following program was used for thermal cycling: 94 °C for 3 min, 55 °C for 2 min, 72 °C for 2 min, then 30 cycles at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1.5 min and a final extension at 72 °C for 10 min. A *c.* 1.5-kb the PCR product obtained with SalI-cut DNA, which contains the mini-Tn5 sequences flanked by genomic DNA, was purified using PCR Clean-Up Kit (Promega), cloned into the pGEM-T Easy vector (Promega) and subjected to sequencing. Determination of the transposon-insertion site was performed with the BLASTN search algorithms (http://www.ncbi.nml.nih.gov/blast/).

Isolation of AHL-minus mutants

A spontaneous mutant of strain C48, resistant to rifampicin, was used as the recipient in mating with *E. coli* S17-1 carrying the pUT-miniTn5-Km2*lacZ* plasmid using a previously described procedure (de Lorenzo & Timmis, 1994). Mutants that were deficient in AHL production were selected on selective LA supplemented with rifampicin and kanamycin plates and tested for their ability to produce AHLs in an on-plate assay using *C. violaceum* CV026 as the reporter. From about 5000 Km-resistant/rifampicin-resistant clones, one clone deficient in AHL-signal production, designated AHL-4, was selected for further work.

Expression of *splIR* genes in *E. coli* and complementation of AHL-4 mutant

For recloning of the *splIR* genes from pGEM-T Easy to the broad-host-range vector pUCP26 (West *et al.*, 1994), both the hybrid plasmid pGEM-T-*splIR* and the vector pUCP26 were restricted with EcoRI. Subsequently, the vector and the insert were ligated and transformed into DH5α. The tetracycline-resistant clones were checked for their ability to produce AHL signals detected by the CV026 reporter on-plate and the presence of the *splIR* insert was verified by restriction analysis and PCR with the same end primers used for cloning into the pGEM-T Easy vector. For complementation of the AHL-4 mutant, the pUCP26-*splIR* plasmid was at first transferred by electroporation into the helper strain *E. coli* S17-1, then used for the conjugal mobilization of pUCP26-s*plIR* plasmids to the AHL-4 recipient strain. The transconjugants were selected on LA plates with rifampicin and tetracycline and verified for the presence of the *splIR* genes and production of AHLs as described above. The pUCP26 vector plasmid was also transferred into the AHL-4 mutant for control.

Pyrrolnitrin extraction and detection

Crude extract of the antibiotic from cells grown on plates with 925 agar medium supplemented with 2% glycerol was prepared by chloroform extraction as described (Ovadis *et al.*, 2004). The amount of Prn obtained after evaporation of the chloroform and dissolution in acetonitrile was measured by HPLC (Spectrum Chromatography, Houston, TX) with a 'Luna' (Phenomenex, Torrance, CA) reverse-phase C18 column (5 μm, 250 × 4.6 mm), eluted isocratically (45% H_2O, 30% acetonitrile, 25% MeOH) at a flow rate of 1 mL min^{-1}. Antibiotic absorption was monitored with a diode-array detector (UV6000) at 225 nm, the wavelength commonly used to monitor pyrrolnitrin (Corbell & Loper, 1995). Purified pyrrolnitrin (Sigma) was used as a standard.

Nucleotide sequence accession number

The GenBank accession number for genes *splI* and *splR* from strain HRO-C48 is AY841161.

Results

AHL pattern of strain C48

The AHL signals produced by strain C48 were detected using the AHL reporter strain *C. violaceum* CV026, specific for AHLs with side chains ranging from four to eight carbons in length (McClean *et al.*, 1997), and the *lux* reporters *E. coli*/pSB536 and *E. coli*/pSB401, highly sensitive to C4-homoserine lactone (butanoyl-homoserine lactone, BHL) and 3-*O*-C6-homoserine lactone (3-oxo-hexanoyl-HSL, OHHL), respectively (Winson *et al.*, 1998). On-plate assays and TLC analysis demonstrated that strain C48 is a strong producer of AHL molecules and the pattern of AHL production consisted of at least three spots with different migration by TLC and sensitivity to the used reporters. With

the CV026 reporter, the production of violacein was strongly observed in a plate-based streak-test assay with strain C48 (data not shown) while in the TLC assay AHL production was observed when the crude ethyl-acetate extract of AHL molecules obtained from 50 mL of the stationary-phase culture of strain C48 grown in LB broth was diluted up to 10-fold (Fig. 1a, line 4) and even 10 000-fold (Fig. 1c, line 2). This response was much higher than that observed with the nontypical *S. plymuthica* strain RHV1 (using 10-fold more of the stationary-phase culture for extraction of AHL molecules), while no AHL signal was observed with this reporter with the *S. plymuthica* DSM30121 (ATCC 183)-type strain (Van Houdt *et al.*, 2005), or with strains *S. plymuthica* IC1270 (Ovadis *et al.*, 2004) or IC14 (Kamensky *et al.*, 2003) (data not shown). The results of the AHL-profile TLC analysis with the CV026 reporter revealed AHLs with different migration patterns. The Rf values of the major upper and minor bottom spots corresponded to the Rf values of OHHL and C6-homoserine lactone (hexanoyl-HSL, HHL) standards (Fig. 1a, line 4). Band corresponding to BHL was not obviously observed in extracts from strain C48 using the CV026 reporter probably being masked by a large spot corresponding to OHHL. However, BHL signal was detected with the *lux* reporter pSB536 (Fig. 1b), which is highly sensitive to this short unsubstituted AHL (Winson *et al.*, 1998). The production of OHHL by strain C48 was also confirmed with the *lux* reporter pSB401, a spot corresponding to OHHL standard in both its Rf value and its typical tail shape was observed with crude extracts diluted up to 10 000-fold (Fig. 1c), indicating that OHHL is the major AHL species produced by this bacterium. No signals were detected by the biosensors when acetonitril was applied to the TLC plates as a control.

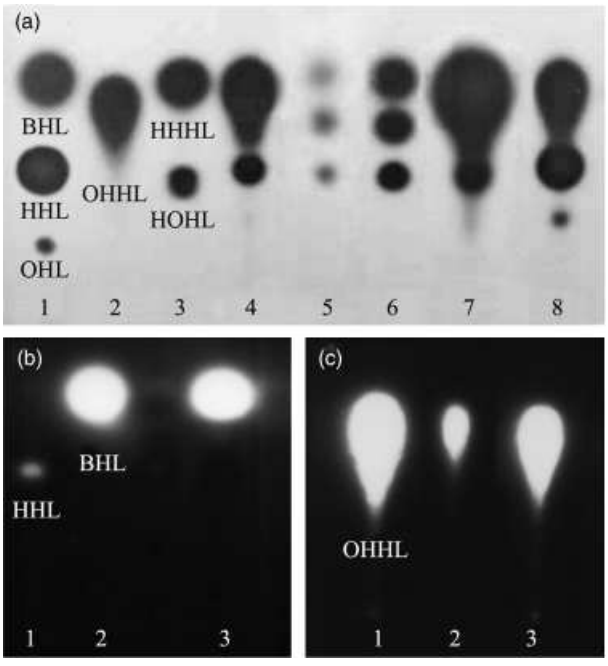

Fig. 1. TLC assay of strain HRO-C48's ability to produce AHLs with *Chromobacterium violaceum* CV026 reporter (a), *Escherichia coli*/pSB536 *lux* reporter (b) and *E. coli*/pSB401 *lux* reporter (c). (a) 1, mixture of BHL (0.5 μg), HHL (50 ng) and OHL (80 ng); 2, OHHL (50 ng); 3, mixture of HHHL (0.5 μg) and HOHL (0.5 μg); 4, extract from strain C48 (crude extract diluted 10-fold, 1 μL); 5 and 6, mutant AHL-4 (1 μL and 5 μL of crude extract, respectively); 7, AHL-4/pUCP26-*splIR*-C48 (crude extract diluted 10-fold, 0.5 μL); 8, DH5a/pGEM-T-*splIR*-C48 (2 μL). (b) 1, HHL (1.2 μg); 2, BHL (0.5 μg); 3, C-48 (crude extract, 5 μL). (c) 1, OHHL (2 ng); 2, C-48 (crude extract diluted 10 000-fold, 5 μL); 3, C-48 (crude extract diluted 1000-fold, 2 μL).

Isolation of AHL-4 mutant and detection of *splIR* genes

To identify genes responsible for AHL production, strain HRO-C48 was subjected to insertion mutagenesis using the *E. coli* strain S17.1 carrying plasmid pUT-miniTn5Km2*lacZ* as the transposon donor. The insertion in the selected mutant AHL-4 was mapped as described in 'Materials and methods'. 'Results' revealed that miniTn5-Km2*lacZ* was inserted in a sequence with a high level of homology to available sequences of *luxI*-like genes *spnI*, *smaI*, *sprI* and *sppI* from various *Serratia* strains and species (GenBank nos. AF389912, AY275980, AY040209 and AY394723, respectively). For complete sequencing of the corresponding genes in strain C48, two primers [5′-TTTGTAGAATACCGGCAAGCTGTT (forward) and 5′-AGATCGTCACGGAGCCTGT (reverse)] were designed matching the end parts of the sequence AY394723 of *luxIR*-like genes *sppIR* (= *splIR*) from strain RVH1 (Van Houdt *et al.*, 2005). PCR and cloning in the pGEM-T Easy vector were repeated with these end primers, and the resultant clone was subjected to sequencing of both strands of inserted DNA, totaling 1441 bp. The genes identified in this region were designated *splI* and *splR* (GenBank accession number AY841161) by analogy with the corresponding *luxI* and *luxR*-family genes from other Gram-negative bacteria (Waters & Bassler, 2005). DNA sequence alignment indicated that the insertion of the miniTn5-Km2*lacZ* transposon in the AHL-4 mutant occurred 220 kb downstream of the ATG start codon of the *splI* open reading frame (Fig. 2). Subsequent sequence comparison with other available *luxIR*-like genes from *Serratia* species revealed that genes *splIR* from strain HRO-C48 are identical with the respective genes from the *S. plymuthica* strain RVH1 (GenBank no. AY394723.2), and very similar to genes *sprIR* from *Serratia proteamaculans* (AY040209) and *spnIR* from *S. marcescens* (AF389912). The deduced SplI protein sequence revealed 83% and 68% identity with the SprI and SpnI proteins, respectively, while the deduced SplR protein from HRO-C48 was 86% and 79% identical to the SprR and SpnR proteins from *S.*

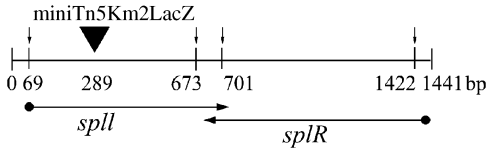

Fig. 2. Gene structure of the *splIR* locus from *Serratia plymuthica* C48 and its mutant AHL-4. The organization of the two regulatory genes, *splI* and *splR*, are shown by arrows indicating position of ATG start codons at 69 and 1422 bp, and corresponding stop codons at 673 and 701 bp, respectively. The exact base pairs are written and indicated by vertical lines. The site of insertion of the miniTn5-Km*lacZ* transposon is indicated as a triangle. Tentative scale is drawn.

proteamaculans and *S. marcescens*, respectively. This phylogenetic comparison supports previous evidences for the horizontal transfer of LuxIR-like systems between *Serratia* species (Wei & Lai, 2006). Moreover, at least in *S. marcescens*, this transfer was shown to be afforded by a transposon structure, which includes the *spnIR* genes (Wei *et al.*, 2006).

The pattern of AHL production by *E. coli* strain DH5α carrying *splIR* genes cloned in the pGEM-T Easy vector appeared similar to that of the parental C48 strain, except for the appearance of an additional spot with an Rf value < 0.30 but higher than 0.16, which corresponded to *N*-3-hydroxyoctanoyl-homoserine lactone (HOHL) and C8-homoserine lactone (OHL) standards (Fig. 1a, line 8). These results indicated that the cloned *splI* gene is responsible for the AHL pattern of strain HRO-C48. Surprisingly, the AHL-4 mutant *splI*::miniTn5 was found to be leaky in terms of AHL production as residual violacein production could still be observed in an on-plate test with the CV026 reporter (data not shown). On TLC plates, the residual activity appeared in a pattern that was quite different from that of the parental strain C48. Three spots with Rf values of 0.62, 0.50 and 0.38, approximating the migration of BHL, OHHL and HHL standards, were visible (Fig. 1a, line 5). However, the upper spot apparently represents 3-hydroxy-C6-homoserine lactone (HHHL) with an Rf value close to 0.60 as well, but not BHL, as this spot was also visible with *A. tumefaciens* NTL4/pZLR4, which is unable to detect BHL (Shaw *et al.*, 1997) (data not shown). The middle spot at Rf 0.50 does not have the tail typical to 3-oxo-homoserine lactone compounds (Shaw *et al.*, 1997) and therefore may represent C7-acyl-homoserine lactone [*N*-(3-hydroxy-heptanoyl)-L-HSL (HHpHL)] with an Rf value of around 0.47. The most slowly migrating spot may represent HOHL rather than HHL. This suggestion is further supported by the fact that in contrast to C48, the AHL signals produced by the mutant AHL-4 are only visible when high amounts of undiluted extract was applied to the TLC plates (see for comparison Fig. 1a, lines 4, 5 and 6). Introduction of the pUCP26-*splIR* plasmid into the AHL-4 mutant completely restored its AHL pattern to that of the parental strain C48 (Fig. 1a, line 7). This result strongly suggests that a high level of SplIR/AHL signal production is normally masked in strain C48 by other AHL molecules to which the CV026 reporter is much less sensitive.

Deficiency in pyrrolnitrin production by the AHL-4 (*splI*::miniTn5) mutant and complementation of the mutant phenotype

The presence of *prn* genes in genomic DNA of strain C48 was first tested by PCR assay with primers to the *prnC* gene of the *prnABCD* operon of *P. fluorescens* (GenBank accession no. U74493), which revealed an expected band around 720 bp. Production of the antibiotic was tested by TLC and HPLC analyses as well as in bioassays using *Rhizoctonia solani*. In a TLC assay, the antibiotic produced by strain C48 and the pyrrolnitrin standard migrated with the same Rf value of 0.76 and this compound exhibited characteristic blue spots after spraying with Ehrlich reagent (data not shown). HPLC analysis of the fraction yielded a peak with a retention time of approximately 31.7 min and an absorption maximum at 225 nm corresponding to purified pyrrolnitrin standard (Fig. 3a).

Contrary to the parental strain C48, its mutant AHL-4 was found deficient in pyrrolnitrin production. Only a very low peak with a retention time corresponding to the pyrrolnitrin standard appeared in the HPLC analysis (Fig. 3b). However, the ability to produce pyrrolnitrin could be completely restored to that of the wild type by introduction of the pUCP26-*splIR* plasmid, but not the pUC26 vector alone, into the AHL-4 mutant (Fig. 3d and e). In addition, the ability of AHL-4 mutants to produce pyrrolnitrin could

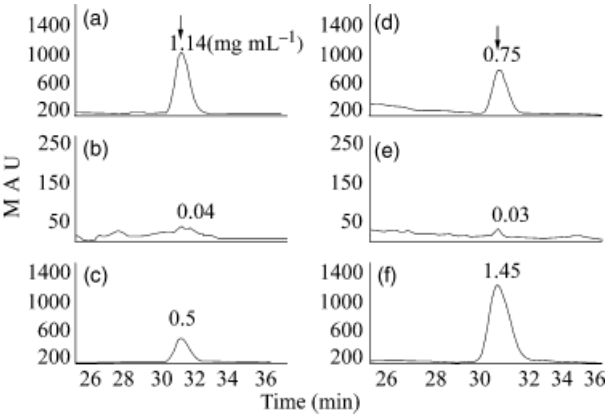

Fig. 3. Detection of pyrrolnitrin production by HPLC analysis. HPLC elution profiles of the crude extract (absorbance at 225 nm vs. retention time in min) isolated from *Serratia plymuthica* C48 and its derivatives are presented. (a), C48; (b), mutant AHL-4; (c), AHL-4 grown in the presence of OHHL (100 μM); (d), AHL-4/pUCP26-*splIR*; (e), AHL-4/pUC26; (f), AHL-4 grown in the presence of cell-free supernatant (10% v/v) of strain AHL-4/pUCP26-*splIR*. Arrow indicates peak yielded by pyrrolnitrin standard. Concentrations of pyrrolnitrin (mg mL^{-1}) in crude extracts are indicated above corresponding peaks.

be at least partially restored by growing the strain on 925 glycerol medium supplemented with chemically synthesized OHHL (100 mM) (Fig. 3c). Moreover, the deficiency in pyrrolnitrin production could be complemented by growing AHL-4 cells on agar medium supplemented with 10% cell-free supernatant from AHL-4/pUCP26-*splIR* cells grown for 24 h at 30 °C in LB (Fig. 3f), while no such effect was observed when cell-free supernatant of strain AHL-4/pUCP26 was used for complementation of the mutant phenotype. Taken together, these results show that strain C48's ability to produce pyrrolnitrin is related to its ability to produce AHLs.

Discussion

This is the first study to demonstrate that pyrrolnitrin production in a bacterium, namely *S. plymuthica*, is regulated by a QS system. The results show that the AHL pattern of strain C48 generally corresponds to that in other *Serratia* strains and species. The AHL signals synthesized by different *Serratia* AHL synthases (LuxI homologues) are mainly represented by the same three AHLs produced by strain HRO-C48. Thus, SmaI and SwrI AHL synthases are responsible for the production of BHL and HHL, SpnI for BHL, OHL, OHHL and HHpHL, and SprI for OHHL (Eberl *et al.*, 1999; Thomson *et al.*, 2000; Horng *et al.*, 2002; Christensen *et al.*, 2003; Rice *et al.*, 2005; Van Houdt *et al.*, 2005).

The AHL-4 mutant was shown to be nearly, but not completely, deficient in AHL production due to insertion of a miniTn5 transposon into the *splI* gene. The residual AHLs could be detected in nondiluted ethyl-acetate extracts of AHL-4 but the pattern of these signals appeared quite different from that of the parental strain. The putative signals can be tentatively attributed to 3-hydroxy-substituted AHLs (see 'Results' for more details), although a precise characterization of these molecules is still required. The existence of other AHLs with the same TLC migration cannot be excluded at this stage; however, introduction of cloned *splIR* genes into the AHL-4 mutant restores its AHL pattern to that of the parental strain C48.

The AHL-4 mutant was found deficient in pyrrolnitrin production. However, the ability to produce pyrrolnitrin could be restored by introducing the pUCP26-*splIR* plasmid or by growth of the AHL-4 mutant cells in the presence of conditioned medium from AHL-4/pUCP26-*splIR* cells. Moreover, this mutant's ability to produce pyrrolnitrin could be at least partially restored by growing the strain on medium supplemented with chemically synthesized OHHL (100 μM). The complementation effect of conditioned medium was higher than that of OHHL, suggesting that in addition to OHHL other AHLs are present in cell-free culture supernatants of strain C48 that might be involved in the regulation of pyrrolnitrin biosynthesis. Obviously, the AHL signals residually produced by the mutant strain when the SplIR system is switched off are, for the most part, not involved in pyrrolnitrin production. However, bacteria can possess multiple QS pathways and further experiments are needed to evaluate if these or QS mimics play a role in the regulation of pyrrolnitrin production by *S. plymuthica* strains *in vitro* and in the rhizosphere. Additional work will also be required to investigate whether SplR controls expression of *prn* genes directly by binding in the promoter region of the operon or by modulating expression of a yet unidentified regulator.

Acknowledgements

This research was supported in part by the National Natural Science Foundation of China (NSFC), Grant Nos. 30370954 and 30670030, as well as by The United States–Israel Binational Agricultural Research and Development Fund (BARD), Grant No. US-3789-05.

References

Ausubel FM, Brendt R, Kingston RE, Moore DD, Seidman JG, Smith JA & Struhl K (1994) *Current Protocols in Molecular Biology*, John Wiley & Sons Inc., New York.

Berg G (2000) Diversity of antifungal and plant-associated *Serratia plymuthica* strains. *J Appl Microbiol* **88**: 952–960.

Chin-A-Woeng TFC, Bloemberg GV & Lugtenberg BJJ (2003) Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *N Phytol* **157**: 503–523.

Christensen AB, Riedel K, Eberl L, Flodgaard LR, Molin S, Gram L & Givskov M (2003) Quorum-sensing-directed protein expression in *Serratia proteamaculans* B5a. *Microbiology* **149**: 471–483.

Corbell NA & Loper JE (1995) A global regulator of second metabolite production in *Pseudomonas fluorescens* Pf-5. *J Bacteriol* **177**: 6230–6236.

Coulthurst SJ, Kurz CL & Salmond GP (2004) *luxS* mutants of *Serratia* defective in autoinducer-2-dependent 'quorum sensing' show strain-dependent impacts on virulence and production of carbapenem and prodigiosin. *Microbiology* **150**: 1901–1910.

de Lorenzo V & Timmis KN (1994) Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Meth Enzymol* **235**: 386–405.

Eberl L, Molin S & Givskov M (1999) Surface motility of *Serratia liquefaciens* MG1. *J Bacteriol* **181**: 1703–1712.

Frankowski J, Berg G & Bahl H (1998) Mechanisms involved in the antifungal activity of the rhizobacterium *Serratia plymuthica*. *IOBC Bull* **21**: 45–50.

Frankowski J, Lorito M, Schmid R, Berg G & Bahl H (2001) Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48. *Arch Microbiol* **176**: 421–426.

Haas D & Defago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* **3**: 307–319.

Horng YT, Deng SC, Daykin M, Soo PC, Wei JR, Luh KT, Ho SW, Swift S, Lai HC & Williams P (2002) The LuxR family protein SpnR functions as a negative regulator of *N*-acylhomoserine lactone-dependent quorum sensing in *Serratia marcescens*. *Mol Microbiol* **45**: 1655–1671.

Kalbe C, Marten P & Berg G (1996) Members of the genus *Serratia* as beneficial rhizobacteria of oilseed rape. *Microbiol Res* **151**: 4400–4433.

Kamensky M, Ovadis M, Chet I & Chernin L (2003) Soil-borne strain IC14 of *Serratia plymuthica* with multiple mechanisms of antifungal activity provides biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* diseases. *Soil Biol Biochem* **35**: 323–331.

Kurze S, Dahl R, Bahl H & Berg G (2001) Biological control of soil-borne pathogens in strawberry by *Serratia plymuthica* HRO-C48. *Plant Dis* **85**: 529–534.

Levenfors JJ, Hedman R, Thaning C, Gerhardson B & Welch CJ (2004) Broad-spectrum antifungal metabolites produced by the soil bacterium *Serratia plymuthica* A 15. *Soil Biol Biochem* **36**: 677–685.

Loh J, Pierson EA, Pierson LS III, Stacey G & Chatterjee A (2002) Quorum sensing in plant-associated bacteria. *Curr Opin Plant Biol* **5**: 285–290.

Mavrodi OV, Gardener BBM, Mavrodi DV, Bonsall RF, Weller DM & Thomashow LS (2001) Genetic diversity of *phlD* from 2,4-diacetylphloroglucinol-producing *fluorescent pseudomonas* spp. *Phytopathology* **91**: 35–43.

McClean KH, Winson MK, Fish L, *et al.* (1997) Quorum sensing in *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **143**: 3703–3711.

Ovadis M, Liu X, Gavriel S, Ismailov Z, Chet I & Chernin L (2004) The global regulator genes from biocontrol strain *Serratia plymuthica* IC1270: cloning, sequencing, and functional studies. *J Bacteriol* **186**: 4986–4993.

Raaijmakers JM, Vlami M & de Souza Jorge T (2002) Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek* **81**: 537–547.

Rice SA, Kjelleberg S, Givskov M, De Boer W & Chernin L (2004) Detection of bacterial homoserine lactone quorum sensing signals. *Molecular Microbial Ecology Manual*, 2nd edn. (Kowalchuk GA, de Bruijn FJ, Head IM, Akkermans ADL & van Elsas JD, eds), pp. 1629–1649. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Rice SA, Koh KS, Queck SY, Labbate M, Lam KW & Kjelleberg S (2005) Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *J Bacteriol* **187**: 3477–3485.

Shaw PD, Ping G, Daly SL, Cha C, Cronan JE Jr, Rinehart KL & Farrand SK (1997) Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc Natl Acad Sci USA* **94**: 6036–6041.

Simon R, Priefer U & Pühler A (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology* **1**: 784–791.

Thomson NR, Crow MA, McGowan SJ, Cox A & Salmond GP (2000) Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. *Mol Microbiol* **36**: 539–556.

Van Houdt R, Moons P, Jansen A, Vanoirbeek K & Michiels CW (2005) Genotypic and phenotypic characterization of a biofilm-forming *Serratia plymuthica* isolate from a raw vegetable processing line. *FEMS Microbiol Lett* **246**: 265–272.

Van Houdt R, Moons P, Jansen A, Vanoirbeek K & Michiels CW (2006) Isolation and functional analysis of *luxS* in *Serratia plymuthica* RVH1. *FEMS Microbiol Lett* **262**: 201–209.

Waters CM & Bassler BK (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21**: 319–346.

Wei JR & Lai HC (2006) *N*-Acylhomoserine lactone-dependent cell-to-cell communication and social behavior in the genus *Serratia*. *Int J Med Microbiol* **296**: 117–124.

Wei JR, Tsai YH, Horng YT, Soo PC, Hsieh SC, Hsueh PR, Horng JT, Williams P & Lai HC (2006) A mobile quorum-sensing system in *Serratia marcescens*. *J Bacteriol* **188**: 1518–1525.

West SE, Schweizer HP, Dall C, Sample AK & Runyen-Janecky LJ (1994) Construction of improved *Escherichia–Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **148**: 81–86.

Winson MK, Swift S, Fish L, Throup JP, Jørgensen F, Chhabra SR, Bycroft BW, Williams P & Stewart GSAB (1998) Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol Lett* **163**: 185–192.